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13. SUPPLEMENTARY NOTES

14. ABSTRACT Nitric oxide (NO) transfer reactions between protein and peptide cysteines are thought to represent a regulated signaling process. In the pulmonary endothelium, endothelial nitric oxide synthase is required for the formation of S-nitrosothiols whereas, S-nitrosoglutathione reductase (GSNO-R) is involved in S-nitrosothiol breakdown. Interestingly, both proteins are regulated by sex steroids: eNOS activity is upregulated by estrogen and GSNO-R is downregulated by testosterone. Previous studies suggest that the differences in GSNO-R activity may be responsible for the gender dependent effects seen in our model of pulmonary hypertension. Examination of S-nitrosothiol hemoglobin content in blood taken from the right ventricle from male and female C57BL6 animals show no differences, suggesting in health, the activities of eNOS and GSNO-R are balanced. Molecularly, we have determined that these two proteins interact, either directly or indirectly and treatment with an S-nitrosylating agent disrupts this interaction. In addition, both eNOS and GSNO-R are S-nitrosylated and overexpression of GSNO-R in endothelial cell culture modifies phosphorylation of eNOS serine 1177, which has been implicated in eNOS activation. Pulmonary responses to eNOS knockout mice demonstrated elevated right ventricular pressures only with SNOAC and Hypoxia. GSNO-R deficient mice fail to respond to any treatment. Taken together, this suggests the existence of an S-nitrosylation/denitrosylation coupling loop and importance on the activity of GSNO-Reductase. Disruption of this loop may lead to the development of pulmonary disease.

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None provided.

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Introduction:

Pulmonary arterial hypertension (PAH), high blood pressure within the lung, is a progressive disease which is characterized by an increase in pulmonary arterial pressure and the formation of muscle around normally non-muscular small pulmonary arteries. Without treatment, PAH progresses rapidly to right heart failure and death. The mechanism(s) sensing the initiating event and transducing this signal into changes in protein expression to alter pulmonary physiology are unclear. The role S-nitrosothiols (SNO) play in the development of PAH is examined in this research project. In the pulmonary circulation, erythrocytes deliver SNOs to recipient target proteins on the surface of the endothelium as a function of oxygen saturation. In this context, erythrocytes can act as a molecular switch, monitoring changes in oxygen saturation to deliver SNOs to the vascular endothelium. We have developed a model in which N-acetyl cysteine (NAC) is used as a tracer to 1) monitor SNO formation, transfer and metabolism *in vivo,* 2) address the physiological and pathological consequences of SNO signaling in the pulmonary vasculature, and 3) identify SNO target proteins in this signaling pathway. Differences in SNO formation, transfer and metabolism and the role this pathway plays in gender specific differences associated with the development of PAH are examined. Studies for this grant period have focused on 1) defining the physiological pulmonary responses of S-nitrosothiols in female mice using our NAC model; 2) analyzing the physiological responses to S-nitrosothiols using mice deficient and null for proteins known to be involved in the formation and metabolism of S-nitrosothiols and 3) identifying proteins and protein/protein interactions in the endothelium that are involved in this pathway.

Body:

S-nitrosothiol Bioavailablity and Gender

Gender is an important risk factor in the development of PAH (1). Human females are twice as likely to get this disease as males (1, 2). Chronic hypoxia can cause PAH in humans (3) and animals (4-9) and as such, can be used as a model system to examine the gender differences associated with the development of this disease. In humans, there is no apparent difference in pulmonary artery pressures seen between male and female children and adolescents living at high altitude (10). However, menarchal humans are better able to adapt to high altitude than males and non-menarchal females (11, 12) suggesting the response to hypoxia can be modulated by sex hormones. Similarly, female rats (13, 14) and swine (15) develop less severe PAH in response to chronic hypoxia compared to males. We have **previously** shown that: 1) chronic administration of N-acetyl cysteine (NAC) elicits PAH indistinguishable from that induced by chronic hypoxia in male C57BL6/129SvEv mice (16). This effect of NAC is not seen in female C57BL6SvEv mice (Figure 1).

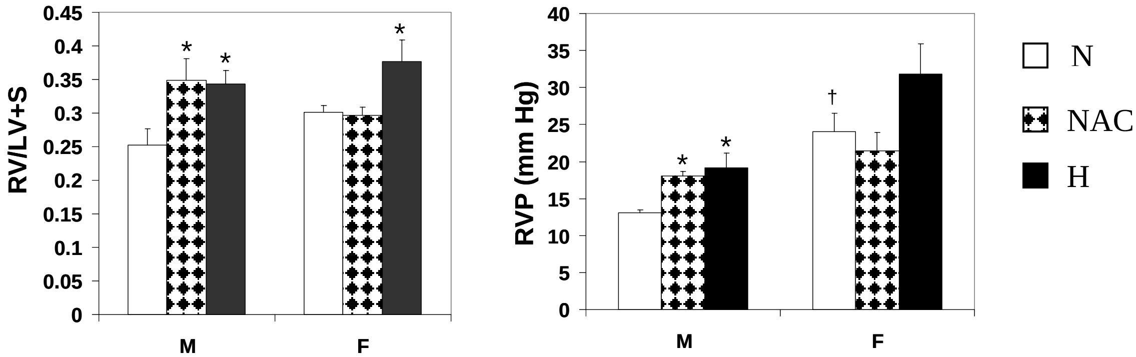

Figure 1. N-acetyl cysteine induces changes in right ventricular weight and pressure in male but not female C57BL6/129SvEv mice. Mice were untreated (N) or treated with 10mg/ml N-acetyl cysteine (NAC) or hypoxia (H) for a period of 3 weeks. (A) Right ventricular pressure (RVP), right heart weight (expressed as right ventricular weight/left ventricular weight + septum weight (RV/LV+S) were determined in male and female C57Bl6/129SvEv mice. Basal (untreated) right heart weight and RVP are greater in female animals. * Significant increases compared to basal levels $p<0.05$. † Significant increases compared to male $p<0.05$.

2) NAC is S-nitrosylated forming SNOAC in the plasma (16) and the levels of SNOAC found in the plasma of female animals is significantly less than SNOAC levels found in the plasma obtained from male animals (Figure 2).

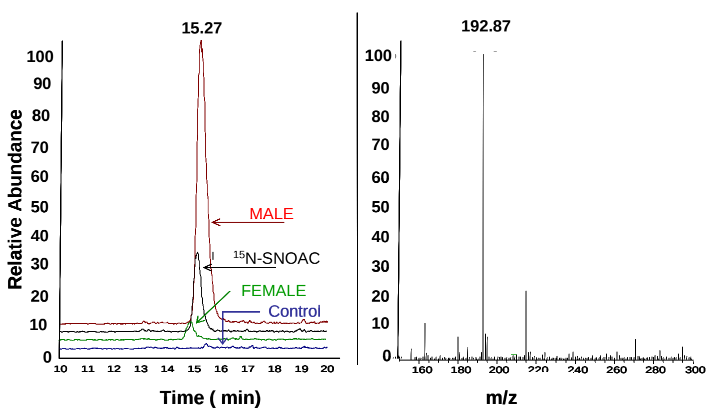

Figure 2. The amount of S-nitrosylated N-acetyl cysteine formed in the serum of NAC treated mice is less in female animals. Serum SNOAC was measured by mass spectroscopy (MS) in C57BL6/129SvEv male and female mice treated with NAC. Left panel = LC chromatogram: Right panel= MS spectrum. Serum from NAC-treated male (red) and female (green) mice had a SNOAC peak (m/z 193) that comigrated with the ^{15}N-SNOAC standard (black). No signal was seen in non treated mice (blue). It should be noted that the amount of SNOAC detected was much less in the female mice.

3) GSNO-R activity in female mice is greater than that seen in males with no significant differences in protein expression (Figure 3).

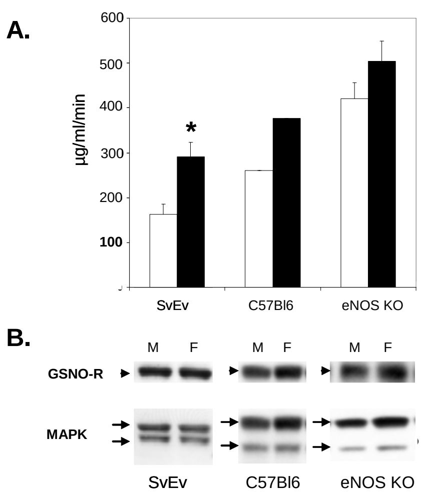

Figure 3. Activity of GSNO-reductase (GSNO-R) is greater in female mice. (A.) GSNO-R activity is measured in male and female C57Bl6SvEv (SvEV), C57Bl6 and eNOS knockout (eNOS KO) mice. GSNO (35µM) was incubated with 500 ng/µl of lung homogenate supplemented with NADH (300 µM), GSH (2mM), ascorbic acid (500 µM), DTPA (100 µM) and PBS pH 7.4. After incubation at 37^{o}C for 5 minutes the enzyme activity was quenched with trichloroacetic acid (5%) and GSNO determined by mass spectrometry. GSNO-R activity in the lung homogenates obtained from female mice was **elevated** compared to male mice in the C57Bl6/129SvEv (n=4) and C57Bl6 (n=2) animals. GSNO-R activity was elevated in the eNOS KO mice compared to the C57Bl6 strain matched controls. In addition, this gender difference was not seen in the eNOS KO animals (n=4). (B) Western blot demonstrating that protein expression of GSNO-R is similar in male and female mice of each strain of animal. Equal loading of the samples was determined by MAPK.

Thus, gender differences in S-nitrosothiol bioavailability may be responsible for the gender discordance seen in the development of PAH.

SNO-bioavailability in the pulmonary vasculature is determined, in part, by the activities of two proteins, endothelial nitric oxide synthase (eNOS) and GSNO-Reductase (GSNO-R). In health, female animals have elevated eNOS expression/activity compared to males due to the production of estrogen (3, 17). This increase in eNOS activity could potentially result in greater SNO production/bioactivity compared to males. However, preliminary data indicate that female animals also appear to have higher GSNO-R activity compared to male mice (Figure 3). In theory, elevated SNO production (increase eNOS activity) in female animals could be balanced through increased GSNO-R activity (increased SNO breakdown), equalizing the SNO content between male and female animals. Analysis of total SNO levels in whole blood taken from the left ventricle of untreated male and female mice demonstrate no significant differences in SNO/hemoglobin (Hb) content (Table 1).

Table 1. SNO/Hb levels are similar in untreated Male and Female C57Bl6/129SvEv Mice

Gender	SNO/Hb
Male	$7.75 \times 10^{-5} \pm 2.49 \times 10^{-5}$
Female	$8.75 \times 10^{-5} \pm 1.50 \times 10^{-5}$

Right ventricular blood was collected from male (n=4) and female (n=4) mice and subjected to reductive chemiluminescence in the presence of carbon monoxide (18). SNO values were normalized to hemoglobin content.

The data suggest that the production and the metabolism of endogenously produced SNOs are balanced in untreated healthy animals.

Imbalances in SNO bioavailability on the functioning of the pulmonary vasculature can be examined in vivo using eNOS deficient (low SNO production) and GSNO-R deficient (decreased SNO turnover) mice. Both strains of deficient mice are on a C57Bl6 background. C57Bl6 animals were obtained from Jackson Laboratories. eNOS knockout (eNOS$^{-/-}$) breeding pairs were originally obtained from Victor Laubach

(University of Virginia) while GSNO-R knockout (GSNO-R$^{-/-}$) breeding pairs were obtained from Jonathan Stamler (Duke University). Studies using the C57Bl6 and eNOS$^{-/-}$ animals have been completed and are presented below (Figures 4-5). However, the two GSNO-R$^{-/-}$ breeding pairs obtained in December 2007 produced no progeny. On the advice of the veterinarian, we backcrossed these original animals to C57Bl6 animals to obtain younger GSNO-R heterozygous (GSNO-R$^{+/-}$) animals. Our first litters were born in October and were genotyped. GSNO-R$^{-/-}$ animals obtained from these crosses were used to set up new breeding pairs. To date, we have 5 breeding pairs of GSNO-R$^{-/-}$ animals, all of which have produced litters. Animals (GSNO-R$^{+/-}$ and GSNO-R$^{-/-}$) not used for breeding purposes were used in our proposed studies. All data obtained from these animals to date are presented (Figures 4, 5): Data obtained for GSNO-R$^{+/-}$ female mice have been completed. Data obtained for GSNO-R$^{+/-}$ male mice are near completion. Data obtained for GSNO-R$^{-/-}$ male and female mice have just begun, and we anticipate completion by the end of March.

Changes in right ventricular weight and right ventricular pressure were measured in C57Bl6, eNOS$^{-/-}$, GSNO-R$^{+/-}$ and GSNO-R$^{-/-}$ animals (Figures 4, 5). Basal right ventricular weights were not different in C57BL6, eNOS$^{-/-}$ or GSNO-R$^{+/-}$ female animals (Figure 4). Although the number of animals (GSNO-R$^{-/-}$) analyzed to date are small (Normoxia: N=2; NAC: N=2, Hypoxia: N=1), it appears that GSNO-R$^{-/-}$ animals have increased basal right ventricular weight compared to C57Bl6 littermates. All of the female animals show an increase in right ventricular weight with hypoxia. No changes in right ventricular weight with NAC or SNOAC treatment were noted. In contrast to the right ventricular weight data, basal right ventricular pressure measurements differ between the genotypes. While there is no significant difference in basal right ventricular pressure between C57Bl6 and eNOS$^{-/-}$ female mice, there does appear to be a gene dosage effect between GSNO-R genes and right ventricular pressure. Increases in right ventricular pressure are seen with SNOAC and hypoxia in C57BL6 and eNOS female mice. These increases are not seen in either the GSNO-R$^{+/-}$ or the GSNO-R$^{-/-}$ female animals examined so far.

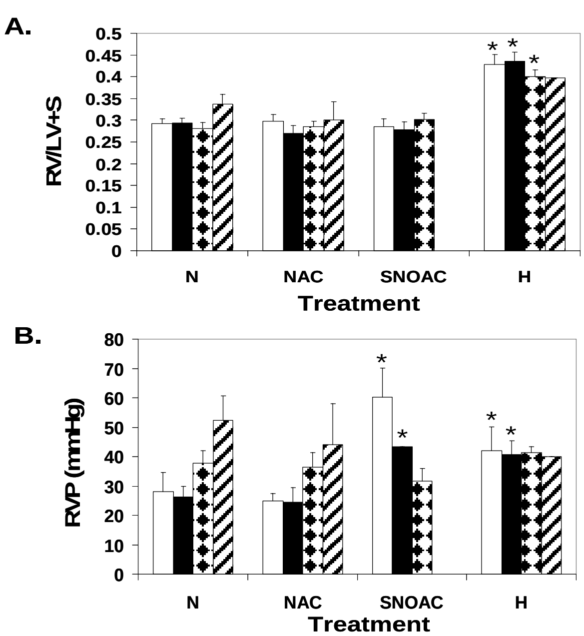

Figure 4. Right Ventricular Weights and Right Ventricular Pressures in Female Mice in Genotypes that affect S-nitrosothiol Bioavailability. C57Bl6 (white bars), eNOS-/- (black bars), GSNO-R+/- (checkered bars) and GSNO-R-/- (striped bars) mice were treated with 10mg/ml NAC, 1mg/ml SNOAC, or 10% oxygen (H) or left untreated (N) for a period of 3 weeks. (A) Right ventricular weight (expressed as right ventricular weight/left ventricular weight + septum weight (RV/LV+S) and (B) right heart pressure (RVP) were determined. P<0.05 compared to normoxic control.

C57BL6 GSNO-R$^{+/-}$
eNOS $^{-/-}$ GSNO-R$^{-/-}$

In summary, all female mice respond to hypoxia with a change in right ventricular weight. Loss of eNOS (eNOS$^{-/-}$) or a deficiency in GSNO-R (GSNO-R$^{+/-}$) does not alter right ventricular weight compared to wild type (C57BL6) control animals, nor does it elicit a change in right ventricular weight upon treatment with NAC or SNOAC. Preliminary data indicate that female GSNO-R null (GSNO-R$^{-/-}$) mice appear to have elevated right ventricular weight in untreated animals compared to the strain matched control animals. This may suggest that GSNO-R plays a role, either directly or indirectly, in the determination of right ventricular weight. The role it plays in NAC, SNOAC or hypoxia remains to be determined. Similarly, all female mice respond to hypoxia with a change in right ventricular pressure. However, wild type (C57BL6) and eNOS$^{-/-}$ mice do not show increases in right ventricular pressure with NAC but do respond to SNOAC. This lack of response to NAC may be due to the increased activity of GSNO-R (Figure 3). The ability of these animals to respond to SNOAC may be due to the ability of SNOAC to over-ride the activity of GSNO-R. The data obtained to date on the GSNO-R animals is less clear due to the small number of animals studied. Right ventricular pressure data from the GSNO-R deficient (GSNO-R$^{+/-}$) and GSNO-R null (GSNO-R$^{-/-}$) suggest a relationship between GSNO-R and right ventricular pressure as there appears to be a gene dosage effect. At present GSNO-R$^{-/-}$ animals do not appear to respond to NAC nor hypoxia, this may be due to the fact that these animals already have elevated S-nitrosothiol levels at baseline due to their inability to degrade S-nitrosothiols.

Our previous data demonstrated that C57Bl6/129SvEv male mice developed PAH in response to NAC, SNOAC and hypoxia (16). C57BL6 male mice responded similarly with increases in right ventricular pressure with NAC, SNOAC and hypoxia (18, Figure 5). Right ventricular weights were also elevated, but not significantly in C57BL6 animals treated with NAC (16). Animals deficient in eNOS (eNOS$^{-/-}$) only responded to SNOAC and hypoxia. This was attributed to the lack of endothelial produced nitric oxide (16). To date, we have almost completed analyzing male GSNO-R deficient (GSNO-R$^{+/-}$) animals(N=5). The data obtained so far are presented in Figure 5.

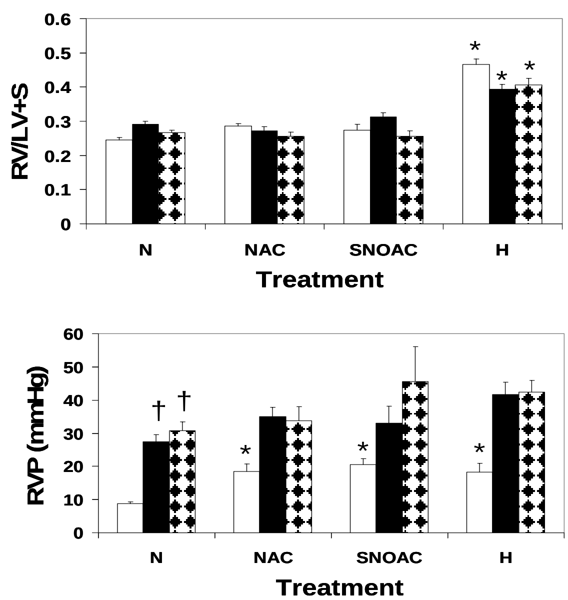

Figure 5. Right Ventricular Weight Right Ventricular Pressures in male mice in Genotypes that affect S-nitrosothiol Bioavailability. C57Bl6 (white bars), eNOS-/- (black bars), and GSNO-R+/- (checkered bars) mice were treated with 10mg/ml NAC, 1mg/ml SNOAC, or 10% oxygen (H) or left untreated (N) for a period of 3 weeks. (A) Right ventricular pressure (RVP), right heart weight (expressed as right ventricular weight/left ventricular weight + septum weight (RV/LV+S) were determined in male and female C57Bl6/129SvEv mice. Right ventricular weight was measured as the weight of the right ventricle (RV) divided by the weight of the left ventricle + septum (LV+S). * P<0.05 ANOVA compared to normoxic control. † p<0.05 compared to wild type

□ C57BL6 ■ eNOS$^{-/-}$ GSNO-R$^{+/-}$

GSNO-R deficient (GSNO-R$^{+/-}$) mice show no changes in right ventricular weight with NAC or SNOAC treatment. Basal right ventricular weight appears to be similar to that seen in eNOS$^{-/-}$ animals. This is elevated compared to the wild type control animals. All animals of different genotypes show elevated right ventricular weights upon exposure to hypoxia. Both eNOS$^{-/-}$ and GSNO-R$^{+/-}$ male animals have elevated right ventricular pressures compared to control wild type (C57Bl6) animals. The response of GSNO-R$^{+/-}$ animals is similar to the eNOS$^{-/-}$ male animals. No GSNO-R$^{-/-}$ animals have been studied to date.

Identification of proteins involved in S-nitrosothiol signaling in the pulmonary vasculature.

Endothelial dysfunction is one the early events that occur in the development of PAH. Endothelial nitric oxide synthase (eNOS) is one protein present in the endothelium and implicated in the development of PAH. S-nitrosylation impacts the expression and/or activity of eNOS. For instance, eNOS is constitutively S-nitrosylated and undergoes rapid denitrosylation upon agonist stimulation (19, 20). However, the ability of eNOS to be S-nitrosylated is dependent on targeting to the plasma membrane (21). Interestingly, local production of NO is associated with the formation of SNOs, which affect protein trafficking (22) and disrupted intracellular membrane trafficking has been suggested to contribute to the development of PAH (23, 24).

Static Culture

The process of S-nitrosylation is a regulated process. Formation and catabolism of S-nitrosylated proteins is balanced. A relationship between eNOS and GSNO-R was suggested by the observation that eNOS$^{-/-}$ mice have elevated GSNO-R activity compared to their strain matched controls (Figure 3) suggesting a relationship between these proteins. Therefore, we explored that relationship in static culture of primary mouse lung endothelial cells (MLECs). Preliminary data indicate: 1) GSNO-R coimmunoprecipitates with eNOS (Figure 10). 2) Exogenous SNO treatment (SNOAC or GSNO) reduces eNOS/GSNO-R interaction and reduces or eliminates its association with caveolin-1 (Figure 10), a protein involved in the regulation of eNOS activity. 3) GSNO-R is S-nitrosylated (Figure 11). 4) Overexpression of GSNO-R results in a slight decrease in total eNOS protein expression as well as a dramatic loss of eNOS phosphorylation at serine 1177 (Figure 12), a modification implicated in eNOS activation.

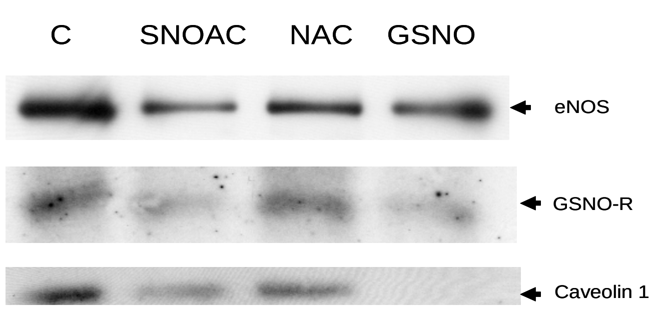

Figure 10. GSNO-R and Caveolin-1 immunoprecipitate with eNOS. Mouse lung endothelial cells were treated with 50µM NAC, 1µM SNOAC or 100µM GSNO or were untreated (C) for a period of 4 hours. Whole cell homogenates were immunoprecipitated with antibody against eNOS. Western blot analysis was performed using eNOS, GSNO-R and caveolin-1 antibodies. GSNO-R immunoprecipitates with eNOS. This interaction is reduced by SNOAC and GSNO. The association with caveolin-1 is reduced or eliminated with SNOAC and GSNO respectively.

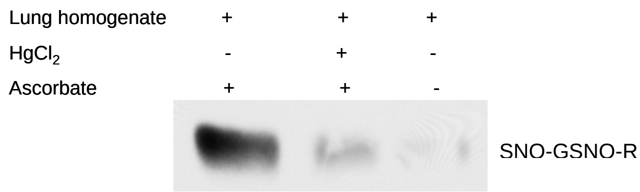

Figure 11. GSNO-R is S-nitrosylated. Lung homogenates from male mice were subjected to the biotin switch protocol in the presence of absence of ascorbate and the presence or absence of mercuric chloride and Western blot performed using GSNO-R antibodies. The appearance of S-nitrosylated GSNO-R (SNO-GSNO-R) was eliminated in the absence of ascorbate or in the presence of mercuric chloride.

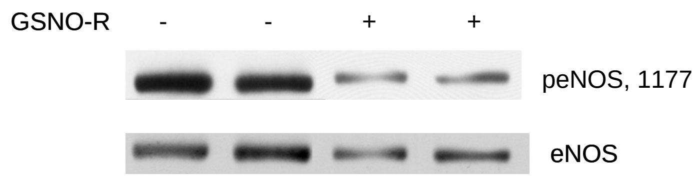

Figure 12. Overexpression of GSNO-R reduces eNOS phosphorylation at serine 1177. Mouse lung endothelial cells were transfected with GSNO-R. Whole cell lysates from transfected cells were subjected to Western blot analysis using antibodies against phosphorylated eNOS (peNOS, residue 1177) and total eNOS. Overexpression of GSNO-R significantly reduced peNOS expression. eNOS expression was also reduced but not to the same extent

The ability of these two proteins, eNOS and GSNO-R, to interact is a novel observation and may impact the activity of eNOS and the development of disease. To further explore the implications of this interaction, we need to determine where within the cell (golgi, vesicles, caveolae, plasma membrane) this interaction occurred. To determine the location of the interaction between eNOS and GSNO-R, a 5-30% discontinuous sucrose gradient was used (25, Figure 13). GSNO-R was found to comigrate with the golgi marker βCOP (fractions 9-12) and was not present in the caveolar enriched fraction (fraction 3-5) suggesting that the interaction between eNOS and GSNO-R is not in the caveolae in cultured cells. The discontinuous sucrose gradients performed in Figure 12 are prepared in sodium carbonate buffer. Sodium carbonate is known to release peripheral membrane proteins from membranes (26). Sodium carbonate extraction was performed to determine if GSNO-R is a peripheral membrane protein (Figure 13).

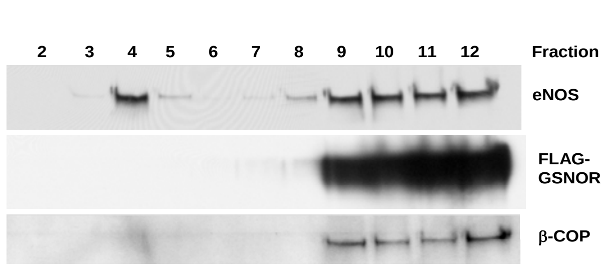

Figure 13. GSNO-R comigrates with the golgi marker βCOP. *Male MLEC were transfected with FLAG-tagged GSNO-R. Cell lysates were separated on a 5-30% discontinuous sucrose gradient (82) to separate caveolar enriched membrane fractions from golgi fractions. 30µl of each fraction were separated on a 12% SDS PAGE and probed for eNOS, FLAG and βCOP(golgi protein marker) and Cav-1 (not shown). FLAG-GSNO-R co localized with βCOP.*

GSNO-R was present in both the cytosolic (supernatant) and membrane (pellet) fractions, suggesting GSNO-R may be a peripheral membrane protein.

The effect of this relationship on the gender differences seen in the development of PAH is not known. However, it is interesting to note that although the association of GSNO-R with the membrane is not determined by gender, treatment with GSNO alters the affinity of this association, which appears to be gender dependent, suggesting that changes in membrane affinity may occur through S-nitrosylation.

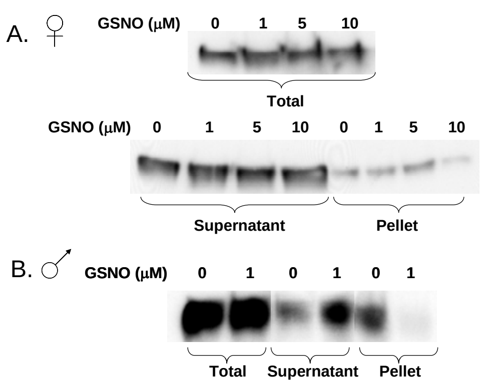

Figure 14. Affinity of GSNO-R for membrane is gender dependent. *Female (A) and male (B) MLECs were transfected with FLAG-tagged GSNO-R, treated with various concentrations of GSNO (4 h) and subjected to sodium carbonate extraction. Similar levels of GSNO-R were expressed in each treatment group (Total). GSNO-R protein was found in both the cytoplasmic fraction (supernatant) as well as the membrane fraction (pellet), suggesting that GSNO-R is a peripheral membrane protein. GSNO treatment with altered the affinity of GSNO-R for the membrane fraction (pellet), which is gender dependent.*

Endothelial/Erythrocyte Fibercell System:

An in vitro model for erythrocyte/endothelial cell interactions using Fibercell technology has begun. This funding period focused on the development a reproducible way to subject the cells grown within the fibercell system to deoxygenation. Conditions used to obtain the hypoxia, changes in pO_2, temperature, pH, pCO_2 were recorded (Table2)

Table 2. Fibercell System, Optimization of Conditions to obtain Hypoxia

Exp	FiO_2	T (C^o)	PO_2	pH	pCO_2	95%N_2/5%CO_2	Time 0f flow (min)	Comments
1	0.21	37	154.4	7.36	39	n/a	n/a	ambient
2	0.21	37	149.0	7.34	42	n/a	n/a	ambient
3	H	36.5	148	7.35	38.4	10kPa	2	Pumped through fibercell, 60x/min
3	H	36	133	7.38	38.5	50kPa	15	
3	H	35	164	7.57	21.8	50kPa	90	
4	H	25	54	-	-	10KPa	5	Bubbled media with $N_2/C0_2$
5	H	25	67	-	-	10KPa	5	Bubbled media with N_2/CO_2
6	H	25	102	6.59	22	10KPa	5	Bubbled media with N_2/CO_2; allowed to sit in room air 1 hour,
7	H	34	65	-	-	10kPa	10min	Bubbled media with N_2/CO_2 on heating pad

Exp= experiment; H= hypoxia

Conditions were measured to determine the optimal conditions required to achieve adequate hypoxia within the fibercell system. Placement of the fibercell system within the tissue culture hood under ambient oxygen levels resulted in similar pO_2 readings (Experiments 1 and 2). Maintenance of hypoxia was examined in experiment 3. In this experiment media was deoxygenated by placing media in portable hypoxia chamber and purging this chamber with 95%$N_2$5%CO_2. Hypoxic media was then pumped through the fibercell system in a tissue culture hood (ambient pO_2) for various time periods. Little change in media pO_2 was seen with time using this method to reduce oxygen content suggesting either 1) that the tubing within the fibercell system is gas permeable, 2) this method of deoxygenation is not sufficient. Changes in pH and pCO_2, however, were noted. In experiments 4 and 5, media was deoxygenated by bubbling 95%$N_2$5%CO_2 through the medium. This effectively reduced the pO2 of the media. Decreases in temperature were noted which can be alleviated by maintaining the temperature of the media with a heating pad (Experiment 7). In experiment 6, media was deoxygenated via the protocol used in experiments 4 and 5 and the length in which this was maintained under ambient pO_2 levels was determined. This confirmed that the tubing within the fibercell system was gas permeable, thus future experiments will be performed in a hypoxic workstation. Lastly, single pass of red blood cells have been tested within the fibercell system. This resulted in little lysis, however, complete removal of the red blood cells from the fibercell system will have to be considered.

From these studies we have determined that the hypoxic exposures will need to be performed in a hypoxic work station. Our initial experiments will examine endothelial luminal surface proteins that will act as SNO-recipient proteins after transfer from the red blood cell. S-nitrosylated proteins on the endothelium will be compared in SNO-depleted and SNO-loaded red blood cells. Two conditions will be tested, single pass of the red blood cell and pulsetile flow. Jaffery analysis will be performed directly within the fibercell. Currently our plan to isolate luminal surface plasma membrane will use positive charged colloidal silicia (27). S-nitrosylated proteins will be identified by mass spectroscopy.

Key Research Accomplishments:

Specific Aim 1: Aberrant formation/transfer and/or delivery of S-nitrosothiols (SNOs) leads to the development of Pulmonary Hypertension

Task 3: Identify the pathophysiolgical changes caused by NAC/SNOAC treatment in the pulmonary vasculature in vivo.

a. Measure the same physiological parameters (changes in right heart weight, right ventricular pressure in wild type mice and eNOS knockout mice. (Figure 5 in text).
b. Set up physiological relevant in vitro endothelial culture system using “fibercell technology” to establish normal flow mediated shear stress
    (1) Develop deoxygenation/endothelial cell interface (Table 2 in text)

(2) Identify S-nitrosothiol target proteins on and in the endothelial cells. (Figures 10-12 in text)

Specific Aim 3. Gender differences in pulmonary hypertension arise from an imbalance of SNO formation and metabolism.

Task 1. Measure the pathophysiological parameters consistent with pulmonary hypertension following chronic administration of NAC/SNOAC to female animals for comparison to that measured in males. (Figure 4 in text)

Task 2. Identify differences in SNOC formation/transfer/metabolism between male and female animals.
(Figure 4 and 5 in text)

Reportable Outcomes.

Patent disclosure: Therapeutic Application of the Effects of Androgens on S-nitrosothiol metabolism in pulmonary disease. L. A. Palmer and B. Gaston

Abstract:
Palmer LA, Brown-Steinke K, de Ronde K, Que L, Gaston B. S-nitrosylation/denitrosylation coupling and the Regulation of Endothelial Nitric Oxide Synthase. Submitted to American Thoracic Society International Conference to be held in San Diego CA May 15-20, 2009.

New Grant applications:
R21 National Institutes of Health: Gender, GSNO-Reductase and the Development of Pulmonary Hypertension: Resubmitted November 2008 for Review in February 2009.

Conclusions
Women are more likely than men to develop PAH (1, 3). However, the molecular basis for this gender difference is not known. Abnormal blood SNO levels have been implicated in the development of this disease. Components (eNOS expression and activation, oxygen affinity of red blood cells, and GSNO-R activity) necessary in the formation, transfer and metabolism of endogenously produced SNOs are generally greater in females than in males. Our preliminary data suggest that the expression and/or activities of GSNO-R may be the key since GSNO-R associates with eNOS; overexpression of GSNO-R alters eNOS phosphorylation at serine 1177, a residue implicated in eNOS activation; castration prevents the development of PAH in response to unregulated delivery of SNOs; and GSNO-R activity in castrated mice is equal to that of female mice. The mechanism by which GSNO-R alters eNOS function and the role that it may play in the gender differences seen in PAH are not known. In the experiments presented above, we have uncovered novel regulatory process which we believe to regulate eNOS activity. We have modified our hypothesis as follows: GSNO-R regulates eNOS activity through S-nitrosylation/denitrosylation coupling, forming a local compartmentalized regulatory loop. Future experiments will include defining the

location of this interaction, the other proteins involved in this pathway, and gender difference involved in this regulation. These new targets may be used to aid in the identification of individuals susceptible to develop this disease as well as lead to the discovery of new therapeutics for the treatment of this disease.

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THERAPEUTIC APPLICATIONS OF THE EFFECTS OF ANDROGENS ON S-NITROSOTHIOL METABOLISM IN PULMONARY DISEASE

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made in part with United States Government support under grants awarded by the National Institutes of Health and the Department of Defense (Army). The United States Government has certain rights in the invention.

BACKGROUND

Marked gender discordance has been observed for decades in the incidence, morbidity and mortality associated with human pulmonary diseases. For example, among children, asthma is more common in boys; however, in adults, nearly 2/3 of patients with asthma are women. In the case of cystic fibrosis, overall mortality among women is nearly 1-2 years earlier than that of men, suggesting that female gender is a risk factor for more severe disease. In the case of pulmonary arterial hypertension of most causes, women are overall more susceptible than men. The cause of this gender discordance is not well understood.

SUMMARY OF THE INVENTION

The present application provides novel data regarding a previously unrecognized mechanism by which there is gender discordance in lung disease—specifically, androgen-induced inhibition of S-nitrosoglutathione reductase *in vivo*. More specifically, the present application discloses that androgens have a specific effect to inhibit S-nitrosothiol breakdown in the lung; this effect can counterbalance estrogen-induced excess S-nitrosothiol production by endothelial nitric oxide synthase and appears to be protective against asthma and cystic fibrosis. This novel finding introduces a novel set of therapies for asthma, cystic fibrosis and pulmonary arterial hypertension involving nebulation of S-nitrosothiol signaling with the use of androgen inhibitors.

There is a balance between cellular S-nitrosothiol breakdown that is co-regulated by androgens and estrogens and appears to be disordered in many patients—particularly women—with pulmonary arterial hypertension. The present invention encompasses the use of specific agonists and androgen mimetic effects—given by inhalation and/or systemically—for the treatment of asthma, cystic fibrosis, pulmonary arterial hypertension and related lung diseases.

The activity of the enzyme, S-nitrosoglutathione (GSNO) reductase, is inhibited in lung homogenates of male mice relative to female mice. Castration of the mice results in an increase in activity; and administration of dihydrotestosterone a decrease in the activity. These are activity effects more than effects on protein expression.

GSNO reductase activity regulates cellular levels of S-nitrosoglutathione. GSNO, in turn, is responsible for airway and vascular smooth muscle relaxation, as well as increased expression, maturation and function of mutant and wild-type cystic fibrosis transmembrane regulatory proteins. An increase in S-nitrosoglutathione therefore reverses the adverse effects of many CF-associated mutations, improves airway hydration and relaxes pulmonary vascular smooth muscle. Therefore, the effect of androgens, dihydrotestosterone, that we observed to inhibit GSNO reductase activity will increase GSNO levels in the lung, and will provide a novel treatment option for asthma, cystic fibrosis and key pulmonary arterial hypertension.

However, chronic, long-term, unregulated exposure to high levels of S-nitrosothiols can result in upregulation of genes that cause pulmonary remodeling. This is particularly true in the case of pulmonary arterial hypertension. There is a balance between S-nitrosothiol production by endothelial nitric oxide synthase and S-nitrosothiol breakdown by S-nitrosoglutathione reductase. In women, it is known that there is increased endothelial nitric oxide synthase expression in the lung. The same has been observed in female mice. This increased expression is balanced by lack of inhibition of GSNO reductase (lack of androgen). Our data suggest that when there is inadequate compensation for increased eNOS expression by decreased (androgen depletion-induced) GSNO-R activity, pulmonary vascular remodeling and pulmonary arterial hypertension will result. In this case, inhibition of androgen signaling in the lungs of female patients with progressive pulmonary arterial hypertension can be a treatment that will prevent upregulation of GSNO-associated remodeling genes, preventing the progression of pulmonary arterial hypertension in women.

In one embodiment, the present invention encompasses the use of androgens, either by inhalation or systemically, to treat diseases and disorders, including, but not limited to, asthma, cystic fibrosis, or related diseases and acute pulmonary arterial hypertension.

In another embodiment, the present invention encompasses the use of androgen receptor antagonists to treat diseases and disorders, including, but not limited to, progressive pulmonary arterial hypertension caused by excessive eNOS activity/estrogen, high flow states, chronic inflammation or chronic hypoxemia.

In one aspect, the dose and type of therapy can be titrated by measuring circulating or pulmonary GSNO reductase activity. That is, if increased S-nitrosoglutathione reductase activity is needed (for example, for chronic pulmonary arterial hypertension, using androgen depletion or inhibition), the dose of androgen inhibitor can be adjusted based on circulating GSNO reductase activity or GSNO reductase activity in a transbronchial biopsy; similarly, if decreased GSNO reductase is needed (for asthma, cystic fibrosis, acute pulmonary arterial hypertension or related diseases of GSNO deficiency), the GSNO level in the airway, and/or the GSNO reductase activity in circulation or in the lung can be used to titrate the dose of androgen given.

Various aspects and embodiments of the invention are described in further detail below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1, comprising left and right panels, graphically depicts the gender differences in response to NAC indicators of PH. Treatments- Normoxia/untreated; NAC; and Hypoxia. The left panel indicates RV weight and the right RV pressure.

Figure 2, comprising left and right panels, graphically demonstrates that castration eliminates NAC-induced PAH. The left panel indicates RV weight and the right RV pressure. Treatments- Gonad intact; Castrated.

Figure 3 graphically depicts that castration increases GSNO-R activity. The ordinate indicates [GSNO] remaining μM, and the abscissa GSNO-R activity.

Figure 4 is a schematic representation of an NAC model.

Figure 5 is a schematic representation of regulatory pathways involved in pulmonary hypertension and possible mechanisms for regulating the pathways.

Figure 6 is a schematic representation of the possible role of S-nitrosothiols and pulmonary hypertension.

Figure 7 is a schematic representation of the possible role Gender and S-nitrosothiols and pulmonary hypertension.

DETAILED DESCRIPTION

Definitions

In describing and claiming the invention, the following terminology will be used in accordance with the definitions set forth below.

The articles “a” and “an” are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

The term "about,” as used herein, means approximately, in the region of, roughly, or around. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. For example, in one aspect, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 20%.

Marked gender discordance has been observed for decades in the incidence, morbidity and mortality associated with human pulmonary diseases. For example, among children, asthma is more common in boys; however, in adults, nearly 2/3 of patients with asthma are women. In the case of cystic fibrosis, overall mortality among women is nearly 1-2 years earlier than that of men, suggesting that female gender is a risk factor for more severe disease. In the case of pulmonary arterial hypertension of most causes, women are overall more susceptible than men. The cause of this gender discordance is not well understood. The present invention discloses that androgens have a specific effect to inhibit S-nitrosothiol breakdown in the lung; this effect can counterbalance estrogen-induced excess S-nitrosothiol production by endothelial nitric oxide synthase and appears to be protective against asthma and cystic fibrosis. There is a balance between cellular S-nitrosothiol breakdown that is co-regulated by androgens and estrogens and appears to be disordered in many patients—particularly women—with pulmonary arterial hypertension. The present invention encompasses the use of specific agonists and androgen mimetic effects—given by inhalation and/or systemically—for the treatment of asthma, cystic fibrosis, pulmonary arterial hypertension, and related lung diseases.

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We have observed that the exogenous S-nitrosothiol, S-nitroso-N-acetyl-cysteine, enters pulmonary vascular endothelial cells and causes upregulation of genes associated with pulmonary vascular remodeling. Administration to mice *in vitro* and *in vivo* causes these effects. Also, in male mice that are eNOS replete, the S-nitroso-N-acetyl-cysteine causes pulmonary vascular remodeling in pulmonary hypertension. eNOS is necessary for this effect (through transnitrosation reactions occurring in hemoglobin) and that eNOS deficient mice are not susceptible to pulmonary arterial hypertension that is caused by S-nitroso-N-acetyl-cysteine.

Of interest, however, female mice are not susceptible to pulmonary arterial hypertension caused by S-nitroso-N-acetyl-cysteine, though they have higher baseline pulmonary pressures than male mice. If the male mice are castrated, they are also protected from S-nitroso-N-acetyl-cysteine. If female mice are ovariectomized, they remain protected from S-nitroso-N-acetyl-cysteine, suggesting that it is not an increase in estrogen/female sex steroids that causes the protection: rather, it is lack of androgen (see Figures). Indeed, we have shown that androgen replacement in the castrated male mice causes them to be susceptible to pulmonary hypertension.

Preliminary data suggest that the overall mechanism is this (Figure 5). Estrogen increases eNOS expression. eNOS is co localized with S-nitrosoglutathione reductase in pulmonary cells. The absence of androgens counter balances the increased nitrosothiol production associated with increased eNOS expression by being permissive for increased GSNO reductase activity. eNOS allows S-nitrosylation of circulating proteins such as hemoglobin and albumin. These, through transnitrosation reactions, form SNOAC. The SNOAC enters lung cells forming GSNO because of high glutathione levels in the cells. Over the long-term, there is upregulation of GSNO-regulated genes causing the vascular remodeling associated with pulmonary hypertension. Thus, males, who have androgen-induced decreased GSNO reductase activity, are susceptible to the long-term gene regulatory effects of N-acetyl-cysteine and S-nitroso-N-acetyl cysteine and, potentially, other S-nitrosothiols to cause pulmonary hypertension. Normal females are protected by virtue of their increased S-nitrosoglutathione reductase activity.

The mechanism appears to be a final pathway for pulmonary arterial hypertension. Chronic hypoxia can cause excessive offloading of nitrosothiols into the pulmonary vascular endothelium, causing remodeling because of allosteric effects of hemoglobin. Excess inflammation can cause dumping of nitrosothiols into the pulmonary vascular endothelium. High flow states, in which an increased number of circulatory nitrosothiols go through the pulmonary vascular endothelium in time, can cause pulmonary vascular remodeling by nitrosothiol dumping. Women with high eNOS activity who do not have adequate compensatory S-nitrosoglutathione reductase activity are particularly at risk.

Though androgens do not increase GSNO reductase expression, we have recently observed that it inhibits GSNO activity (Figure 1). This inhibition of S-nitrosoglutathione reductase activity would be good for asthma and cystic fibrosis (increasing GSNO levels), it would be good acutely for pulmonary hypertension, leading to decreased pulmonary vascular tone (perhaps suggesting why the female mice have higher pulmonary artery pressures at baseline). However, in the long-term, this excess in nitrosothiols upregulates hypoxia-associated genes.

We have previously published the work on N-acetyl-cysteine and S-nitroso-N-acetyl-cysteine causing pulmonary hypertension in male mice. However, the observation that female mice are protected, and the mechanistic information regarding androgens (castration, androgen replacement, ovariectomy) is novel and forms the basis of the invention.

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2. U.S. Patent: “Inhibiting GSNO Breakdown”

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated by reference herein in their entirety.

Headings are included herein for reference and to aid in locating certain sections. These headings are not intended to limit the scope of the concepts described therein under, and these concepts may have applicability in other sections throughout the entire specification.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention.

ABSTRACT

There is a gender discordance in many human lung diseases, including asthma, cystic fibrosis, and pulmonary arterial hypertension. This discordance affects incidence, morbidity and mortality. We have discovered a novel pathway by which androgens can be both beneficial and can have adverse effects in these lung diseases. This is a completely novel mechanism involving S-nitrosothiol metabolism in the lung.

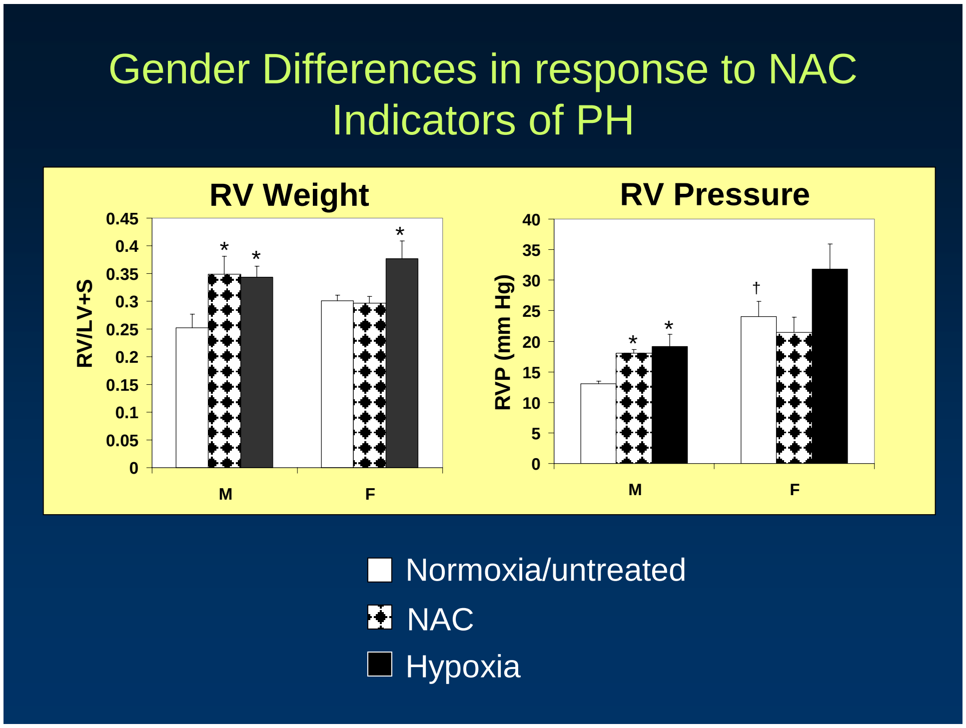

Gender Differences in response to NAC
Indicators of PH
RV Weight
RV/LV+S
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
M
F
RV Pressure
RVP (mm Hg)
40
35
30
25
20
15
10
5
0
M
F
Normoxia/untreated
NAC
Hypoxia

FIG. 1

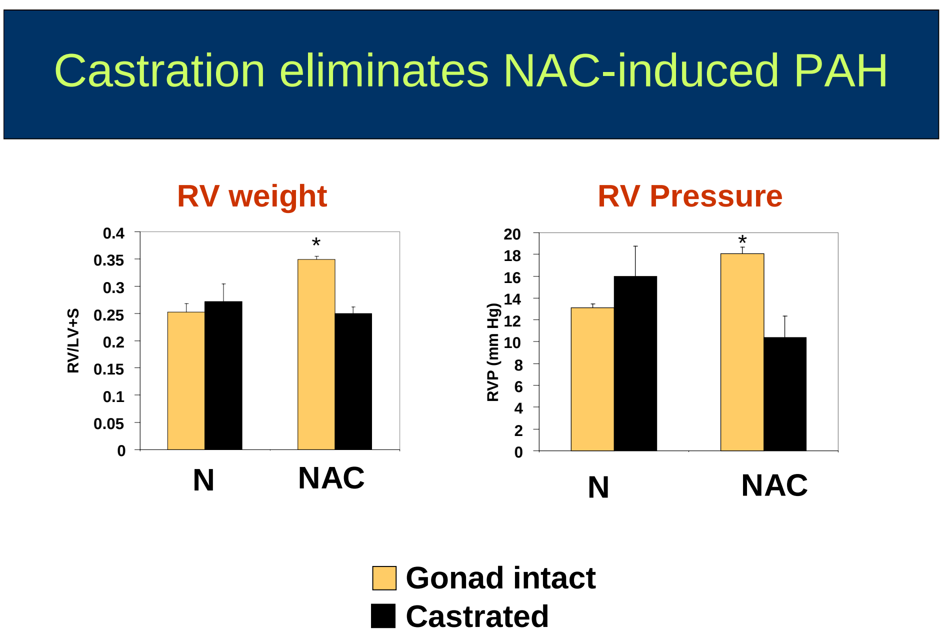
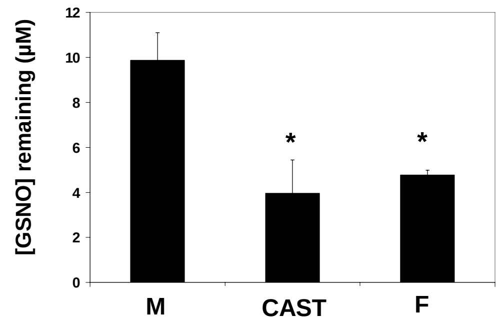

Castration eliminates NAC-induced PAH
RV weight
RV/LV+S
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
N
NAC
RV Pressure
RVP (mm Hg)
20
18
16
14
12
10
8
6
4
2
0
N
NAC
Gonad intact
Castrated

FIG. 2

Castration increases GSNO-R activity



GSNO-R activity

FIG. 3

NAC Model:
Androgen Effects

1. Castration:
   - eliminates development of PH with NAC
     - right ventricular pressure
     - right ventricular weight
   - increases GSNO-R-Activity

2. Ovariectomy:
   - No effects (*data not shown*)

3. GSNO-R is regulated by androgens.

FIG. 4

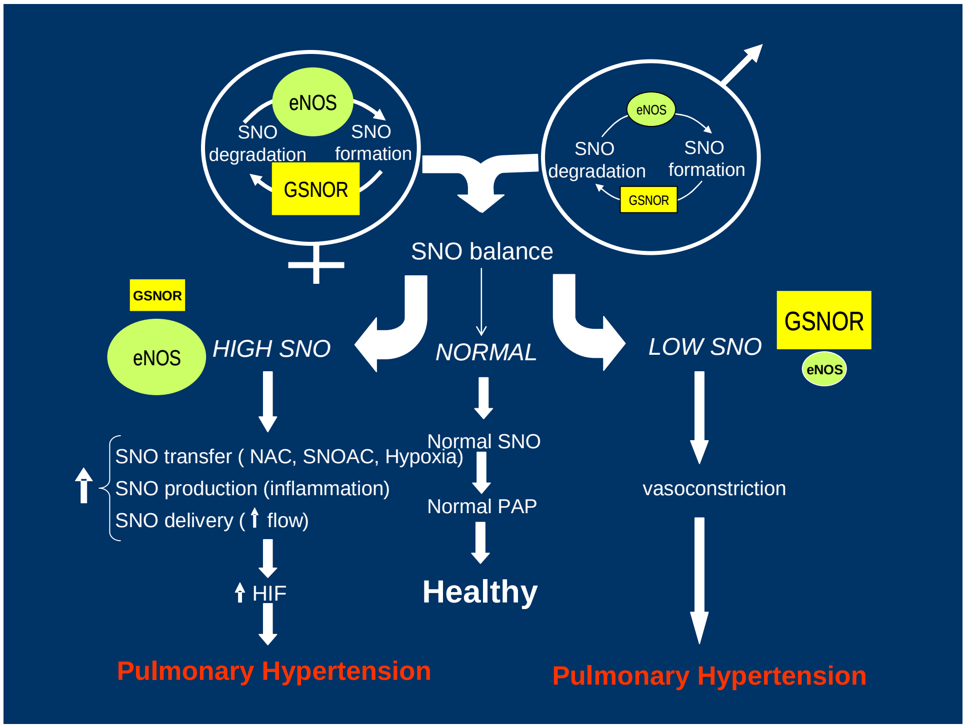

eNOS
SNO degradation
SNO formation
GSNOR
eNOS
SNO degradation
SNO formation
GSNOR
SNO balance
GSNOR
eNOS
HIGH SNO
NORMAL
LOW SNO
GSNOR
eNOS
SNO transfer (NAC, SNOAC, Hypoxia)
SNO production (inflammation)
SNO delivery (flow)
Normal SNO
Normal PAP
vasoconstriction
HIF
Healthy
Pulmonary Hypertension
Pulmonary Hypertension

FIG. 5

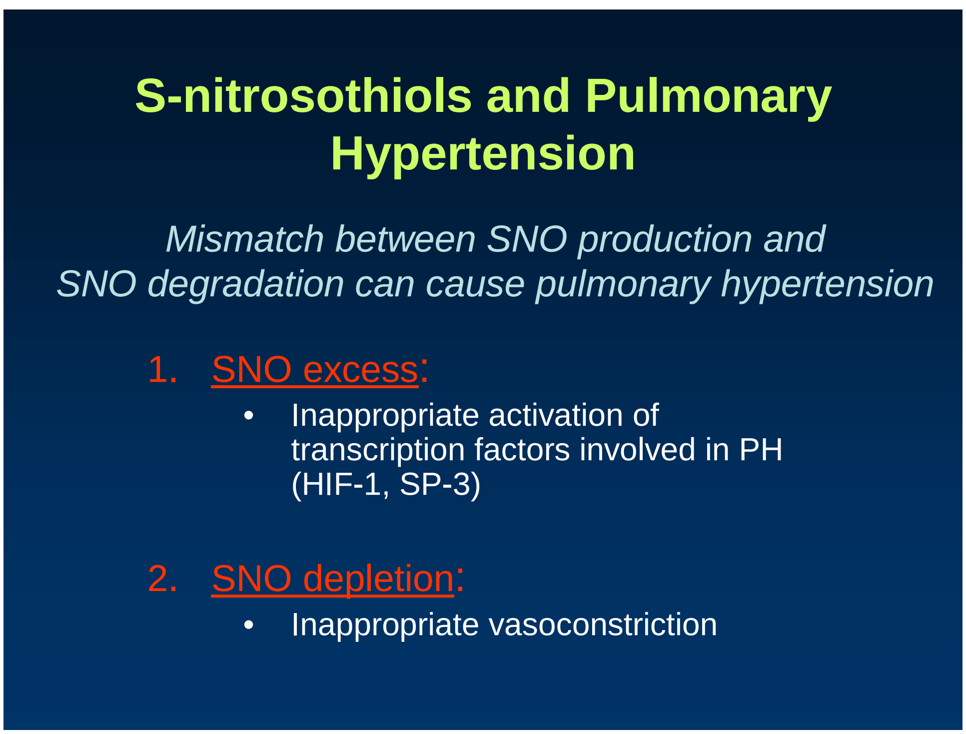

S-nitrosothiols and Pulmonary Hypertension
Mismatch between SNO production and SNO degradation can cause pulmonary hypertension
1. SNO excess:
• Inappropriate activation of transcription factors involved in PH (HIF-1, SP-3)
2. SNO depletion:
• Inappropriate vasoconstriction

FIG. 6

Gender and S-nitrosothiols

SNO Bioavailability may be responsible, in part, for gender disparities.

1. SNO formation:
   - **Estrogen known to regulate eNOS**
2. SNO degradation:
   - **Androgens downregulate GSNO-R activity**
3. SNO excess:
   - **NAC model ⟶ males develop PH**

FIG. 7

S-nitrosylation/denitrosylation coupling and the Regulation of Endothelial Nitric Synthase.

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*Duke University, Durham, North Carolina

The formation of S-nitrosothiols requires nitric oxide synthase (NOS) activation. Endothelial NOS (eNOS) is the primary NOS found in the endothelium. S-nitrosothiol catabolism, in part, requires the action of S-nitrosoglutathione reductase (GSNO-R). Since S-nitrosothiol bioavailability within cells is determined by the balance between S-nitrosothiol formation and catabolism, we examined the relationship between these two proteins. Co-immunoprecipitation studies indicate GSNO-R associated with eNOS in murine pulmonary endothelial cells. Treatment with S-nitrosoglutathione (GSNO), an endogenously produced S-nitrosylating agent, disrupts this interaction. To determine if this interaction alters eNOS activity, murine pulmonary endothelial cells were transfected with GSNO-R in the absence or presence of GSNO. In the absence of GSNO, GSNO-R overexpression decreased the abundance of phosphorylated eNOS at serine 1177, a site associated with eNOS activation whereas, treatment with GSNO reversed the effect. Lastly, the possibility that the interaction between these proteins was regulated by S-nitrosylation was examined using the biotin switch assay. GSNO-R was found to be S-nitrosylated; S-nitrosylation was abrogated by a cysteine to serine mutation at residue 282. Interestingly, eNOS S-nitrosylation was absent in lysates obtained from cells transfected with wild type GSNO-R containing the cysteine to serine mutation. Taken together, the data suggest that the activities and interaction between eNOS and GSNO-R are mediated by S-nitrosylation/denitrosylation coupling and may function to regulate S-nitrosothiols bioavailability within the cells.

Specific Aims:

Pulmonary arterial hypertension (PAH) is a condition with known gender disparity. We propose that sex-linked discrepancies in the bioactivities of S-nitrosothiols (SNOs), which are potent regulators of pulmonary arterial pressure, play an important role in the gender divergence seen in this disease. The vascular bioactivity of SNOs is determined by three components: formation, delivery from the red blood cell, and metabolism. The formation of SNOs is affected by endothelial nitric oxide synthase (eNOS) activation; SNO efflux from the red blood cell is largely determined by oxygen saturation; and SNO metabolism is regulated, in part, by the activity of S-nitrosoglutathione reductase (GSNO-reductase). There are gender differences in all these regulatory mechanisms, which are generally greater in females than in males. Using a novel mouse model of PAH induced by unregulated delivery of SNOs to the pulmonary vasculature, our data reveal female mice had higher resting right ventricular pressures than males, but are protected from the additional SNO-induced increases observed in males. Castration eliminated this difference. GSNO-reductase appears to be fundamental to the observed gender differences since GSNO-reductase activity in castrated mice was equal to that of female mice, and overexpression of GSNO-reductase altered eNOS phosphorylation at serine 1177, a residue implicated in eNOS activation. Based on these observations, **we hypothesize 1) disruption of the eNOS-GSNO-reductase system plays an important role in one gender compared to the other with respect to the development of PAH and 2) disruption of the delicate balance between eNOS and GSNO-reductase activities, which maintain a steady state of SNO bioactivity, contribute to the gender specific differences seen in PAH.**

<u>Specific Aim 1 tests the hypothesis that crosstalk between GSNO-reductase and eNOS contributes to S-nitrosothiol bioactivities seen in the in the pulmonary vasculature.</u>

1a.) Measure the gender differences in the expression and activity of GSNO-reductase in mice.
1b.) Determine the influence of GSNO-reductase on the expression and activity of eNOS.
1d.) Determine the influence of eNOS on the expression and activity of GSNO-reductase.
1c.) Identify the subcellular compartment(s) where GSNO-reductase and eNOS interact

<u>Specific Aim 2 tests the hypothesis that the gender discordance seen in pulmonary arterial hypertension is due to alterations in GSNO-reductase activity.</u>

2a.) Determine the mechanism by which sex steroids alter the expression and/or activity of GSNO-reductase.
2b.) Determine if GSNO-reductase polymorphisms are associated with the development of pulmonary arterial hypertension in humans.

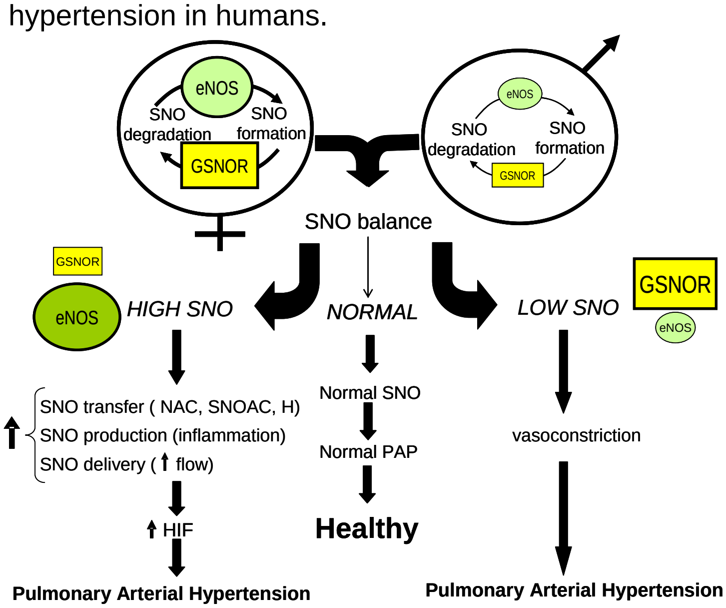

Figure 1. Mismatch between SNO-production/ delivery and SNO-metabolism may contribute to the development of PAH. The importance of maintaining a balance between SNO production and degradation is illustrated in the schematic diagram. The relative expression and/or activity of eNOS and GSNO-reductase are different in male and female mice, with females having increased expression and/or activity of both proteins. An imbalance in SNO bioavailability in the pulmonary vasculature could lead to the activation of downstream events that contribute to the development of pulmonary arterial hypertension. This proposal examines the role GSNO-reductase activity and/or expression plays in the gender discordance seen in this disease.

In summary, we hypothesize SNO bioactivity is regulated by the eNOS-GSNO-reductase system, that sex hormones modulate the activity/expression of both enzymes and that altered SNO bioavailability resulting from a disruption in the balance between eNOS and GSNO-reductase activities causes PAH. Although blood SNO levels are similar in male and female mice, eNOS and GSNO-reductase activities are increased in females. As such, we hypothesize that disturbances in the eNOS-GSNO-reuctases system (ie SNO production/turnover rate), may have a more drastic consequence in females than in males.